recent progress in understanding mechanisms of vesicle fusion, the molecular mechanisms of synaptotagmin C2AB membrane docking remain incompletely characterized. For example, the two C2 domains of Syt1 are reported to cooperatively insert into target membranes, but specific interdomain contacts have not been identified. To test whether the two C2 domains from Syt7 interact on a planar lipid bilayer, lateral diffusion constants of fluorescent-tagged C2A, C2B, and C2AB domains from human Syt7 were measured on PC:PS (3:1) bilayers using total internal reflection fluorescence microscopy with single-particle tracking. The Syt7 C2AB tandem exhibits a lateral diffusion constant half the value of the isolated single domains, and does not change when additional residues are engineered into the C2A-C2B linker. This is the expected result if C2A and C2B are separated when membrane-bound; theory predicts that C2AB diffusion would be faster if the two domains interact. Furthermore, ensemble stopped-flow measurements of membrane dissociation kinetics also support an absence of interdomain interactions, as EDTA-induced dissociation kinetics of the C2AB tandem are similar to the isolated C2A domain and remain unchanged when rigid or flexible linker extensions are included. Together, the results suggest that the two C2 domains of Syt7 bind independently to membranes. Ongoing efforts seek to perform analogous measurements with Syt1, whose C2 domains have much shorter membrane-bound lifetimes.

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Single Molecule Diffusion Studies of PTEN: Insights into Membrane Binding

Rakesh K. Harishchandra[1], Anne-Marie M. Bryant[1], Abigail C. Cornwell[1], Mathias Lösche[2], Alonzo H. Ross[3], Arne Gericke[1].
[1]Department of Chemistry and Biochemistry, Worcester Polytechnic Institute, Worcester, MA, USA, [2]Department of Physics, Carnegie Mellon University, Pittsburg, PA, USA, [3]Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, USA.

PTEN, a tumor suppressor gene that encodes a dual specificity phosphatase that dephosphorylates phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$), is one of the most frequent genes deleted or mutated in a wide variety of tumors. PTEN acts as an antagonist to phosphoinositide-3-kinase (PI3K) signaling, thereby affecting various cellular processes such as cell proliferation and survival. The activity of PTEN is regulated by dynamic shuttling between the cytoplasm and the plasma membrane. The membrane association of PTEN strongly depends on the composition and lateral distribution of the lipids in the membrane. Several biophysical techniques have been used to characterize PTEN-membrane interaction. Here, we use single-molecule total internal reflection fluorescence microscopy (TIRFM), which is a powerful tool to study the molecular mechanism of membrane targeted proteins. The single-molecule TIRFM allows us to observe single PTEN molecules as they dynamically associate/dissociate and laterally diffuse along the lipid bilayer membrane. PTEN lipid binding is investigated on supported lipid bilayers of binary and ternary lipid mixtures of PC with physiological relevant levels of PS, $PI(4,5)P_2$ and PI. We tracked individual PTEN molecules and statistically determine the lateral diffusion and dwell time of PTEN on heterogeneous lipid bilayers. We find significant differences in PTEN dynamic behavior when bound to the different membrane environments. Furthermore, to gain insight into the molecular mechanisms of PTEN membrane association, we compared the lipid binding of wt PTEN, an N-terminally truncated PTEN-(Δ1–15 AA) that lacks the $PI(4,5)P_2$ binding site, and the recently discovered, PTEN-L with a 173 AA N-terminal extension. We find profound differences in the dynamic behavior of these PTEN derivatives at the membrane. It has recently been suggested that PTEN associates as a dimer with the membrane. We find that the tendency to form membrane bound dimers varies among these PTEN variants.

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A Single-Molecule Imaging Based Method for Estimating Subunit Stoichiometry of Purified Membrane Protein Complexes in Liposomes

Rahul Chadda[1], Larry Friedman[2], Ankita Chadda[1], Mike Rigney[2], Luci-Kolmakova Partensky[2], Jeff Gelles[2], Janice L. Robertson[1].
[1]Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA, USA, [2]Department of Biochemistry, Brandeis University, Waltham, MA, USA.

Estimating the number of subunits of a purified novel membrane protein is challenge. There are various methods for determining stoichiometry accurately in detergent, but often we are interested in the protein structure in the native solvent environment - the lipid bilayer. Here we describe a robust and widely applicable method to estimate the number of subunits in a purified membrane protein sample using single molecule fluorescent imaging. First, the multimeric protein complex is solubilized in detergent and purified. Second, it is quantitatively labelled with Cy5-maleimide and reconstituted into liposomes made of E. coli polar lipids or 2:1 POPE/POPG, doped with 0.3% of AF488-NHS ester labelled POPE. Following multiple freeze/thaw cycles to form multilamellar vesicles, the proteoliposomes are extruded through polycarbonate filters of 30, 100 and 400 nm pore diameter resulting in reproducibly distinct size distributions. Reconstitution follows the Poisson distribution resulting in liposomes containing either 0, 1, 2 or more protein molecules. The apparent protein occupancy into liposomes depends on protein:lipid density, efficiency of fluorescent labelling, liposome surface area and the subunit stoichiometry of the protein complex. Liposome size distributions are determined by cryo-electron microscopy whereas the protein density and fluorescent-labeling is controlled during the reconstitution step. We measure the first three terms in the Poisson distribution: F0 (unoccupied liposomes), F1 (single occupancy) and F2 (double occupancy) by single molecule imaging of the fluorescent proteoliposomes to measure protein/lipid co-localization and photobleaching of protein conjugated fluorophores. We test our model with a CLC-ec1 engineered monomeric construct, the native CLC-ec1 homodimer and tetrameric KcsA K^+ channel. The methods outlined in this study can be used to determine the subunit stoichiometry of unknown purified membrane protein complexes in a variety of liposome environments.

Mechanosensation

2840-Pos Board B270

Nanobiomechanics and Mechanotransduction of Sensory Neurons

Laura Andolfi[1], Marco Lazzarino[1], Valentina Masciotti[1], Yanmei Qi[2], Jing Hu[2].
[1]IOM-CNR, Trieste, Italy, [2]Centre for Integrative Neuroscience, Tübingen, Germany.

A detailed knowledge of mechanical parameters such as cell elasticity, stiffness and viscoelasticity is essential for understanding the mechanisms that control the mechanotransduction in mechanosensory neurons (MSN). Indeed, in order to tune and maximize their sensitivity, MSN should be neither too rigid nor to compliant; moreover they are expected to show different elasticity as a function of the typology of mechanical stimulus they should record. However a precise correlation between MSN mechanical properties and mechanotransduction mechanism is still missing, and the sensory mechanical transduction, necessary for the senses of touch and pain, remains poorly understood.
Indentation measurements by atomic force microscopy (AFM) enable to investigate and quantify in vitro the softness of living MSN thanks to its ability to measure low forces (pN) and nanometer scale displacement. Moreover, the integration of AFM with fluorescence microscopy opens up the possibility to relate the involvement or activation of either cytoplasmatic structures or transmembrane proteins with variations of cell mechanical properties and, as result, their role in the modulation of mechanosensory neurons activity.
In this study we performed AFM indentation measurements to evaluate the mechanical properties of wild type and genetically modified proteins of the stomatin system of dorsal root ganglia (DRG). We found a decrease of cell elasticity in DRG neurons where stomatin system is genetically modified. The role of cell elasticity in mechanotransduction regulation of mechanosensory neurons is discussed.

2841-Pos Board B271

Cross-Linked Matrix Rigidity and Soluble Factors Induce Differentiation via Distinct but Overlapping Pathways

Irena L. Ivanovska, Joe Swift, Kyle Spinler, P.C. Dave P. Dingal, **Dennis E. Discher**.
Biophysical Eng'g Lab, University of Pennsylvania, Philadelphia, PA, USA.

Stem cell differentiation is regulated by both soluble factors and the physical properties of extracellular matrix, but the extent to which differentiation pathways are distinct or overlap is often unclear. Here, the micromechanical stiffness of the collagenous bone surface together with broad compositional correlations with collagen-I across many soft tissues suggests enzymatic cross-linking of matrix correlates with nucleoskeletal protein lamin-A, with a retinoid receptor RARG, and with induction toward osteogenesis. Collagen films just 2 nm thick on mica were stiffened or not by transglutaminase cross-linking and used as minimal culture substrates for Mesenchymal stem cells (MSCs). Cells pulling on pristine nano-films visibly deformed and aligned with the collagen fibrils, but on cross-linked films, cells spread isotropically as if adhering to a substrate of greater effective stiffness. Cell nuclei also spread and stiffened, with an increase of lamin-A, nuclear localization of RARG, and upregulation of key early and late osteogenic factors. RARG antagonists also increased lamin-A, and enhanced osteogenesis on rigid substrates in vitro as well as in xenografts of MSCs in mice. A model of the underlying Mechano-chemical Gene Circuit couples the sensitivity of stem cells to both insoluble

and soluble factors, while a proteomic comparison underscores both differences and overlaps in differentiation pathways.

2842-Pos Board B272

Altered Contractile Machinery in Airway Epithelial Cells in Response to Cigarette Smoke

Corrine Kliment, Vasudha Srivastava, Douglas Robinson, **Ramana Sidhaye**.
Medicine, Johns Hopkins University, Baltimore, MD, USA.

Chronic obstructive pulmonary disease (COPD) is the 3rd leading cause of death in the US with cigarette smoke being the primary insult leading to disease progression. While the lung epithelium is the site that makes initial and primary contact with the inhaled cigarette smoke, we do not understand the role of cytoskeleton and cell mechanics in the airway response to cigarette smoke. We know that cytoskeletal proteins, such as myosin II, actin, alpha-actinin, the catenins, and E-cadherin, are involved in cell-cell adhesion formation and barrier function of the lung. Furthermore, E-cadherin levels decrease in primary epithelial cells from COPD patients. Therefore, we hypothesized that cigarette smoke drives key cytoskeletal protein changes that lead to alterations in barrier function and thereby influence the development of chronic lung disease. To test this, we studied major cytoskeletal (actin and nonmuscle myosin II isoforms) and cell adhesion proteins (E-cadherin) in normal human bronchial epithelial cells (NHBE, collected directly from human patients) or 16HBE cells (primary human bronchial epithelial cell line) grown on an air-liquid interface. Using a Vitrocell smoke chamber, we found that in response to acute smoke exposure (four cigarettes in 24 hrs), myosin IIB and actin assembled into apical stress fibers, which are not normally found in airway epithelial cells. Cigarette smoke exposure also resulted in decreased total E-cadherin levels as well as decreased E-cadherin at the basolateral surface. Thus, cigarette smoke promotes dramatic acute cytoskeletal changes that likely lead to altered cell mechanical properties (tension, elasticity), which in turn lead to reduced epithelial barrier function and our data suggest that epithelial barrier function is critical in dictating chronic tissue responses that contributes to COPD development and progression.

2843-Pos Board B273

Primary Cilia Length is Critical to Cellular Mechanotransduction

Milos Spasic, Christopher Jacobs.
Biomedical Engineering, Columbia University, New York, NY, USA.

Mechanotransduction is an essential cellular function in a variety of tissues including bone, kidney, and endothelia. The primary cilium is a single immotile organelle protruding from the surface of these cells, and has repeatedly been demonstrated as a critical mechanotransducer in these cell types. When the primary cilium is impaired, these cells have an abrogated response to mechanical stimulation. Here, we demonstrate a method to enhance cellular mechanotransduction, by increasing primary cilia length. To elongate primary cilia, we treated MLO-Y4 osteocytes with fenoldopam, lithium chloride, or vehicle control for 16 hours. We then subjected these cells to oscillatory fluid flow for 1 hour at 1 Hz and 1 Pa wall shear stress. Immediately following flow, cells were lysed, and mRNA expression was analyzed. Cells with longer cilia displayed increased expression of osteogenic markers cyclooxygenase-2 and osteopontin, compared to vehicle control, suggesting that these cells are more mechanosensitive. To discern the role of fenoldopam on cilia length from other cellular processes, we treated cells with IFT88 siRNA_IFT88 is critical for primary cilia formation_which resulted shorter cilia and an abrogated response to fluid flow. Fenoldopam was able to restore cilia length in cells with impaired cilia formation, and rescued flow-induced osteogenic signaling. Together, these data suggest that cells with longer cilia are more mechanosensitive, and that cellular mechanotransduction can easily be modulated by pharmacologically lengthening primary cilia. Primary cilia-mediated mechanotransduction is a critical function in an array of cell types, and numerous diseases, such as polycystic kidney disease and Bardet-Biedl Syndrome, are characterized by impaired cilia function. This work suggests a potential therapeutic strategy to combat such conditions.

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Structure of an Inner-Ear Protocadherin-15 Fragment with an Atypical Calcium-Free Linker

Raul Araya-Secchi, Marcos Sotomayor.
Chemistry and Biochemistry Department, The Ohio State University, Columbus, OH, USA.

Tip links are protein filaments essential for hearing and balance. They convey force to and gate inner ear hair cell transduction channels to mediate sensory perception. Cadherin-23 and protocadherin-15 form tip links through a calcium-dependent heterophilic interaction of their extracellular domains, which are comprised by multiple modules termed extracellular cadherin ''EC'' repeats. These EC repeats are similar but not identical to each other in terms of sequence and structure, often featuring highly-conserved calcium-binding sites at the linker region between them. Recent sequence analyses and structures of cadherins revealed unusual calcium-free inter-repeat linkers in some protocahderins and other non-classical cadherins. Bound calcium ions have been shown to provide structural rigidity to cadherins, thus the presence of unusual sites may confer higher flexibility and perhaps affect the tertiary and quaternary arrangement of cadherins that harbor them. Analysis of the protocadherin-15 sequence shows unusual calcium-binding sites in some of its inter-repeat linkers. Here we present the x-ray crystal structure of repeats EC8-10 refined at 3.3 Å resolution, which shows an EC9-10 calcium-free linker that alters the linear arrangement of protocadherin-15's EC repeats. We suggest that several unusual features of these repeats affect the overall elastic response of protocadherin-15 that is relevant for tip link function in sensory perception.

2845-Pos Board B275

Force-Free Transition from Closed to Open MscL: A Molecular Dynamics Study

Natalie E. Smith, Ben Corry.
Research School of Biology, The Australian National University, ACT, Australia.

The mechanosensitive channel of large conductance (MscL) is exceptionally important in bacterial cells as when the cellular pressure becomes too high and the membrane tension rises, these channels rapidly open allowing an efflux of cellular contents that prevents the cell from bursting. As there are no homologues of this channel in humans, it is seen as a promising new target for antibiotics, something that is critically important given the increasing resistance to existing drugs. While MscL has been studied for many years, exactly how it senses membrane tension to go from a closed to an open state is still unknown, however, it has been observed that mutations in the gating region strongly affect the tension required to open the channel. We have applied molecular dynamics to study wild type MscL and three channel mutants, G22E, G22S and G22N-G26N that each have lower gating tension thresholds than wild type. In the absence of any external force we have observed specific and interesting differences in the propensity of each channel to transition towards the open state. On the timescale sampled for each mutant channel (0.5-2.25 μs), we have observed sub-conductant states of MscL allowing both the measurement of an appreciable current and atomistic detail of how the transition from a closed to a partially open channel occurs. This provides a model for how mechanical forces can be converted into a physiological response.

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Patch Clamp Characterisaton of the Effect of Cardiolipin on the Bacterial Mechanosensitive Channels of Small (MscS) and Large (MscL) Conductance

Pietro Ridone[1], Yoshitaka Nakayama[1], Boris Martinac[1,2],
Andrew R. Battle[3,4].
[1]Victor Chang Cardiac Research Institute, Darlinghurst, 2010, Australia, [2]St Vincent's Clinical School, The University of New South Wales, Kensington, 2052, Australia, [3]Griffith Health Institute, Griffith University, Gold Coast Campus, 4222, Australia, [4]School of Biomedical Sciences, The University of Queensland, St Lucia, 4072, Australia.

The bacterial mechanosensitive channels MscS and MscL respond to membrane tension by opening when the bacterium experiences hypoosmotic shock conditions to prevent cell lysis [1]. Environmental factors such as cholesterol [2] and cations/anions [3] also affect the gating behaviour of these channels. We have previously shown that addition of the negatively charged lipid cardiolipin to POPE/POPC membranes cause rapid and flickery behaviour of MscS [4]. Here, in an expanded study to include MscL, we compare the gating kinetics and pressure sensitivitiy of the channels with and without cardiolipin in both azolectin and mixtures of pure lipids DOPE/DOPC. In azolectin liposomes, mixtures of 10% cardiolipin abolish hysteresis of MscS, but MscL remains largely unaffected, indicating it may stabilise the closed state of MscS. Compared to the azolectin, mixtures of DOPE/DOPC abolish the hysteresis gating of MscS even in the absence of cardiolipin and addition of cardiolipin increases the opening and closing thresholds of both MscS and MscL. These results suggest that cardiolipin shows a significant effect on the mechanosensitive gating of both MscS and MscL.

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